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(54) Title: TREATMENT OF TUMORS BY GENETIC TRANSFORMATION OF TUMOR CELLS WITH GENES ENCODING NEGATIVE SELECTIVE MARKERS AND CYTOKINES

(57) Abstract

A method of treating a tumor in a host which comprises administering to the host at least one expression vehicle including a first nucleic acid sequence encoding an agent which renders the tumor cells sensitive to an interaction agent, and a second nucleic acid sequence which encodes an agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells, wherein such agent is not an agent which renders tumor cells sensitive to an interaction agent. The tumor then is treated with the interaction agent. Preferably, the first nucleic acid sequence encodes a negative selective marker (such as Herpes Simplex Virus thymidine kinase), and the second nucleic acid sequence encodes a cytokine (such as Interleukin-2), and the first and second nucleic acid sequences are contained in first and second viral vectors, each of which is contained in a first producer cell line and in a second producer cell line, respectively. The producer cell lines are administered to the tumor, whereby viral particles generated by the producer cell lines infect the tumor cells. Upon administration of an interaction agent, such as ganciclovir, to the host, the tumor cells are killed.

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TREATMENT OF TUMORS BY GENETIC TRANSFORMATION OF TUMOR CELLS WITH GENES ENCODING NEGATIVE SELECTIVE MARKERS AND CYTOKINES

This application is a continuation-in-part of application Serial No. 07/877,519, filed May 1, 1992, and incorporated herein by reference in its entirety.

This invention relates to the treatment of tumors. More particularly, this invention relates to the treatment of tumors (such as, for example, ovarian cancer) with DNA (RNA) encoding an agent which renders tumor cells sensitive to an interaction agent and DNA (RNA) encoding a cytokine.

Ovarian carcinoma is the most common cause of death from a gynecological malignancy in the United States with approximately 19,000 newly diagnosed cases per year and a 70% overall mortality rate. Over two-thirds of patients have an advanced stage of the disease at presentation for which systemic chemotherapy is indicated after staging and debulking laparotomy. (Young, et al., "Cancer of the Ovary," in DeVita, et al., eds., Cancer Principles and Practice of Oncology, J.B. Lippincott, Philadelphia, pgs. 1226-1254 (1993)). Although about 80% of patients respond to initial treatment with cisplatin-based chemotherapy, only about 10% to 20% experience durable complete remissions. (Young, et al., 1993.) Paclitaxel, a natural

product, provides a 21% to 50% objective response rate in patients with refractory or recurrent disease. (Taxol Clinical Brochure, The Division of Cancer Treatment, NCI (1983); McGuire, et al., Ann. Intern. Med., Vol. III, No. 4, pgs. 273-279 (1989); Thigpen, et al., Proc. ASCO, Vol. 9, pgs 156 (1990); Einzig, et al., Proc. AACR, Vol. 31, pg. 187 (1990); Sarosy, et al., Proc. ASCO, Vol. II, pg. 226 (1992)). Despite such response rate, patients with relapsed or refractory disease after initial therapy have a uniformly fatal outcome.

Ovarian cancer has a fairly unique natural history. Even patients with advanced stages of the disease often have their diseases confined to their abdomens for extended periods of time. The cancer often stays localized to the abdomen and presents great difficulty for the patient by obstruction of the intestines or ureters. As a result, intraperitoneal therapies have been developed for the local administration of chemotherapeutic agents into the peritoneal cavity. (Meyers, Semin. Oncol., Vol. II, pgs. 275-284 (1984)). These therapies have met with only moderate success because they did not provide the reduced toxicity profiles initially hoped for.

Gene transfer has been recognized for some time as a promising avenue to therapies for cancers, among other diseases. The earliest applications of gene transfer for cancer treatment have been indirect approaches focusing on enhancing anti-tumor immune responses. Thus, for instance, attempts have been made to increase the cytotoxicity of immune cells, or to enhance their proliferation.

In one approach, tumor cells have been modified in vitro with cytokine genes and reintroduced into patients in an attempt to immunize the patient to their own cancer. In animal studies, the IL-4 gene was introduced to tumors by Tepper, et al., Cell 57: 503 (1989); the IL-2 gene by Fearon, et al., Cell 60 :397 (1990), and by Gansbacher, et

al., J. Exp. Med. 172: 1217 (1990); the interferon-gamma gene by Gansbacher, et al., Cancer Res. 50: 7820 (1990); and TNF gene by Asher, et al., J. Immunol. 146: 3227 (1991). Each of the animal studies demonstrated rejection of genetically altered tumors upon reimplantation, and the mice in these studies were immune to subsequent rechallenge with the same tumor.

Ezzeddine, et al., New Biologist 3: 608-14 (1991), have reported on the use of retroviral vector-mediated gene transfer in vitro in an attempt to treat tumors. More specifically, a murine retroviral vector was employed to introduce a thymidine kinase gene from herpes simplex virus 1 ("HSV-1 tk gene") into C6 rat glioma-derived cell lines in vitro. Cells which had taken up the retroviral vector were sensitized to the anti-viral agent ganciclovir, and were preferentially killed when exposed to ganciclovir in the medium.

Ezzeddine, et al. were able to use the method to define conditions in vitro for killing essentially all infected cells but not uninfected cells. In addition, C6 cells were introduced subcutaneously into nude mice to form tumors and the tumor-bearing mice were treated with ganciclovir. Ganciclovir inhibited the growth of tumors formed by HSV-1 tk expressing C6 cells, but did not affect tumors formed by HSV-1 tk-negative C6 cells.

Ezzeddine, et al. thus showed that in vitro retroviral gene transfer can be used to sensitize cells to a cytotoxic agent, which can then be used to kill the cells when they are propagated as tumors in nude mice. The authors did not demonstrate any practical way to introduce an HSV-1 tk gene into tumor cells in situ, however. Ezzeddine, et al. also did not show how to eradicate all neoplastic cells, a prerequisite for tumor remission, when less than all cells in the tumor would take up a tk gene, express the gene at a

level sufficient to assure toxicity and, as a consequence, be killed by exposure to ganciclovir.

Short, et al., J. Neurosci. Res. 27: 427-33 (1990), have described the delivery of genes to tumor cells by means of grafting a retroviral vector-packaging cell line into a tumor. The packaging cell line produced a replication-defective retroviral vector in which the MoMLV LTR promoter-operator was used to drive expression of ß-galactosidase, which served as a marker of retroviral vector propagation. When the packaging cell line was grafted into a tumor, ß-galactosidase expression in situ was seen only in packaging cells and in proliferating tumor cells, not in normal tissue.

Despite the apparent preference for tumor cells, propagation of the retroviral vector from producer cells to tumor cells was relatively inefficient, according to Short, et al., and only a fraction of the cells in the tumor were infected. Furthermore, practically no galactosidase expression was observed when cell-free retroviral vector particles were introduced to a tumor directly rather than in a packaging cell line. Short, et al. opined that a packaging cell line might be used to deliver a "killer" or "suppressor" gene to tumor cells, but observed an efficiency of infection far below what would be required for therapeutic utility based on direct gene transduction into all the cells of a tumor.

A novel approach to treat solid brain tumors by in vivo retroviral-mediated transfer of the Herpes Simplex thymidine kinase gene into tumor cells, which confers sensitivity to the antiviral drug ganciclovir, has been described (Culver, et al., Science, Vol. 256, pgs. 1550-1552 (1992); Ram, et al., Cancer Res., Vol. 53, pgs. 83-88 (1993)). Ganciclovir is phosphorylated preferentially by transduced tumor cells and interferes with DNA synthesis. Gene transfer is achieved by infection or tumor cells with

murine retroviral vectors carrying the Herpes Simplex thymidine kinase gene and integration of this gene into the genome of the host cell. These vectors are produced continuously by murine vector producer cells that are injected into the tumor mass. Because retroviruses can infect only cells that are synthesizing DNA actively (i.e., replicating cells), a preferential transduction of tumor cells is achieved. This approach now is being evaluated in a clinical trial. (Oldfield, et al., Human Gene Therapy, Vol. 4, pgs. 39-69 (1993)).

PCT Application No. W093/04167, published March 4, 1993, discloses a purported method for transferring therapeutic genes to brain tumor cells in order to kill the cells. In such method, a retrovirus containing a selectable marker and at least one gene required for its replication is introduced into producer cells such that integration of the proviral DNA corresponding to the retrovirus into the genome of the producer cell results in the generation of a modified retrovirus wherein at least one of the genes required for replication of the retrovirus is replaced by the therapeutic gene or genes. Producer cells then are selected in which the modified retrovirus is incorporated as part of the genome of the producer cells. The producer cells then are grafted in proximity to the dividing tumor cells in order to infect the tumor cell with the modified retrovirus, thereby transferring the therapeutic gene or genes to the tumor cells. The cells then are killed by administering a substance that is metabolized by the therapeutic gene transferred to the tumor cells into a metabolite that kills the cells. The therapeutic gene may be the Herpes Simplex thymidine kinase gene, and the substance which is metabolized by Herpes Simplex thymidine kinase to kill the tumor cells may be gancyclovir or acyclovir. The cited PCT application shows only (i) that a replication-defective retrovirus which

carried an HSV tk gene and a G418 resistance gene could be transduced stably, via G418 selection, into a glioma cell line in vitro; (ii) that the viral tk gene in the transformed cells rendered them about 20-fold more sensitive to ganciclovir than control glioma cells; and (iii) that some glioma tumor cells which formed tumors when implanted in rat brains also expressed a β-galactosidase marker when the tumors were injected with a producer cell line which produced a retroviral vector with the marker gene. The vector in the described experiments did not carry a tk gene, and there was no systemic administration of a chemotherapeutic agent. Thus, the PCT application in question does not show that tumor cells can be rendered sensitive in vivo to any such agent.

European Patent Application No. 476,953, by Martuza, et al., discloses similar results. This publication also describes replication defective retroviral vectors for expressing an HSVtk gene or a β-galactosidase gene in tumor cells. Vectors were transfected into glioma cells in vitro and stable cell lines that expressed HSVtk activity were cloned out from the transfectants. The cell lines then were implanted into rats and the resulting tumors were found to be more sensitive to ganciclovir than control glioma cell tumors. The results, like those of the foregoing publications, demonstrated that ganciclovir kills cells that express HSVtk activity. The application further discloses the use of producer cells to deliver a replication-defective retroviral vector containing a reporter gene to glioma cells in the rat brain. However, no more than 10% of the tumor cells were transduced.

The Martuza application, like the other documents addressed above, assumes that a vector must proliferate throughout cells in a tumor to achieve a therapeutic effect. It does not suggest a way to effectuate this result in a tumor in situ, however.

A solution to this problem is described in U.S. application serial No. 07/877,591 ("'591 application"). As set forth in the '591 application, a "bystander effect" can occur in tumors *in vivo* whereby, for instance, a sensitizing gene administered to a tumor *in situ* renders some tumor cells sensitive to the effects of an interaction agent, although such cells were not transduced. A viral tk sensitizing gene administered by microinjecting producer cells into a tumor *in situ*, by the bystander effect, renders sensitive to ganciclovir tumor cells which are not transduced to express the viral tk. The '591 application teaches that this effect radically augments the therapeutic efficacy of the sensitizing gene in a way that could not have been predicted.

The bystander effect and its uses in gene transfer therapies are discussed in detail in the '591 application. As noted there, anti-tumor therapies may be potentiated by administering, in addition to the sensitizing gene, a gene that stimulates or activates the immune system, thereby increasing the overall percentage of killed neoplastic cells in the tumor. Among the immune response enhancing genes disclosed in the '591 application are cytokines, including but not limited to IL-1 through IL-12, and immune co-activating signal molecules, such as certain MHC determinants.

The '591 application discloses IL-2 as a particularly preferred cytokine in this regard. The '591 application is not limited to any particular mechanism by which IL-2 activity might augment an anti-tumor effect of a sensitizing gene. Nonetheless, it is hypothesized there that IL-2 and other immune response enhancing genes would improve therapeutic efficacy by stimulating or activating the immune system.

The inventors of the present application have found surprisingly that an IL-2 gene, *inter alia*, augments the

action of a sensitizing gene without immune system activity, using ovarian cancer cells as a model system. As disclosed below, an IL-2 gene in *in vitro* experiments potentiated the ganciclovir sensitivity of cells of an ovarian tumor cell line upon transduction with an HSVtk expression vector. In addition, the synergistic effect of IL-2 and the sensitizing gene was seen *in vivo* in the absence of an immune system in implanted ovarian cell tumors in nude mice. And the potentiating effect of IL-2 on HSVtk/ganciclovir-mediated genetic therapy of fibrosarcoma cell tumors also has been demonstrated in animals with intact immune systems, by implication at least partly by a mechanism that does not operate through the immune system.

Without being limited to any theoretical reasoning, the potentiating effect of Interleukin-2 on an HSVtk/ganciclovir-mediated gene therapy for ovarian cell tumors is indicative of an unusual and unrecognized mechanism of anti-tumor activity of a cytokine.

It is an object of the present invention to provide a method of treating tumors in a host, whereby there is achieved a synergistic tumoricidal effect between thymidine kinase and a cytokine.

In accordance with an aspect of the present invention, there is provided a method of treating a tumor in a host. The tumor is sensitive to the bystander effect. The method also enhances the bystander effect. The host may be a human or non-human animal host. The method comprises administering to the tumor a first nucleic acid sequence, and a second nucleic acid sequence. The first nucleic acid sequence encodes an agent such that the tumor cells are rendered sensitive to an interaction agent; i.e., growth of the tumor cells is inhibited, prevented, or destroyed upon administration of the interaction agent. The second nucleic acid sequence encodes an agent which provides for

the inhibition, prevention, or destruction of the growth of the tumor cells, but is not an agent which renders tumor cells sensitive to an interaction agent. Upon administration of the first nucleic acid sequence and the second nucleic acid sequence to the tumor, the tumor then is treated with an interaction agent. The therapeutic effect of the interaction agent is enhanced by the agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells (but is not an interaction agent), and such effect is an effect independent of the immune system of the host.

The term "nucleic acid sequence" as used herein, means a DNA or RNA molecule, and includes complete and partial gene sequences, and includes polynucleotides as well. Such term also includes a linear series of deoxyribonucleotides or ribonucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of the adjacent pentoses.

The first and second nucleic acid sequences are contained in at least one expression vehicle. The term "expression vehicle" as used herein means any genetic construct including the first and/or second nucleic acid sequences, and is capable of providing for expression of such sequence(s).

In one embodiment, the first nucleic acid sequence is contained in a first expression vehicle, and the second nucleic acid sequence is contained in a second expression vehicle. The expression vehicle may be any expression vehicle which is capable of transfecting cells and expressing the first and/or second nucleic acid sequence(s) in vivo. Such expression vehicles include, but are not limited to, eukaryotic vectors, prokaryotic vectors (such as, for example, bacterial plasmids), and viral vectors. The vector also may be contained within a liposome.

In one embodiment, the first and second expression vehicles are viral vectors. Viral vectors which may be employed include, but are not limited to, retroviral vectors, adenovirus vectors, adeno-associated virus vectors, and Herpes Virus vectors. Preferably, the viral vector is a retroviral vector.

In a preferred embodiment, a first packaging cell line is transduced with a first viral vector, which includes the first nucleic acid sequence which encodes an agent such that the tumor cells are rendered sensitive to an interaction agent, to form a first producer cell line including the first viral vector. A second packaging cell line also is transduced with a second viral vector, which includes the second nucleic acid sequence which encodes an agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells, wherein the agent is not an agent which renders the tumor cells sensitive to an interaction agent, to form a second producer cell line including the second viral vector. The producer cell lines then are administered to the tumor, whereby the producer cell lines generate viral vector particles capable of transducing the tumor cells.

In a preferred embodiment, each of the first and second viral vectors is a retroviral vector. Examples of retroviral vectors which may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus. Preferably, the retroviral vector is an infectious but non-replication competent retrovirus. However, replication competent retroviruses may also be used.

Retroviral vectors are useful as agents to mediate retroviral-mediated gene transfer into eukaryotic cells.

Retroviral vectors generally are constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. Most often, the structural genes (i.e., gag, pol, and env), are removed from the retroviral backbone using genetic engineering techniques known in the art. This may include digestion with the appropriate restriction endonuclease or, in some instances, with Bal 31 exonuclease to generate fragments containing appropriate portions of the packaging signal.

These new genes are incorporated into these vectors by a variety of well-known methods. Perhaps the most straightforward constructions are ones in which the structural genes of the retrovirus are replaced by a single gene which then is transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Retroviral vectors have also been constructed which can introduce more than one gene into target cells. Usually, in such vectors one gene is under the regulatory control of the viral LTR, while the second gene is expressed either off a spliced message or is under the regulation of its own, internal promoter.

Efforts have been directed at minimizing the viral component of the viral backbone, largely in an effort to reduce the chance for recombination between the vector and the packaging-defective helper virus within packaging cells. A packaging-defective helper virus is necessary to provide the structural genes of a retrovirus, which have been deleted from the vector itself.

In one embodiment, the retroviral vector may be one of a series of vectors described in Bender, et al., J. Virol., 61:1639-1649 (1987), based on the N2 vector (Armentano, et al., J. Virol., 61:1647-1650) containing a series of deletions and substitutions to reduce to an absolute minimum the homology between the vector and packaging

systems. These changes have also reduced the likelihood that viral proteins would be expressed. In the first of these vectors, LNL-XHC, there was altered, by site-directed mutagenesis, the natural ATG start codon of gag to TAG, thereby eliminating unintended protein synthesis from that point. In Moloney murine leukemia virus (MoMuLV), 5' to the authentic gag start, an open reading frame exists which permits expression of another glycosylated protein ($pPr80^{gag}$). Moloney murine sarcoma virus (MoMuSV) has alterations in this 5' region, including a frameshift and loss of glycosylation sites, which obviate potential expression of the amino terminus of $pPr80^{gag}$. Therefore, the vector LNL6 was made, which incorporated both the altered ATG of LNL-XHC and the 5' portion of MoMuSV. The 5' structure of the LN vector series thus eliminates the possibility of expression of retroviral reading frames, with the subsequent production of viral antigens in genetically transduced target cells. In a final alteration to reduce overlap with packaging-defective helper virus, Miller has eliminated extra env sequences immediately preceding the 3' LTR in the LN vector (Miller, et al., Biotechniques, 7:980-990, 1989).

The paramount need that must be satisfied by any gene transfer system for its application to gene therapy is safety. Safety is derived from the combination of vector genome structure together with the packaging system that is utilized for production of the infectious vector. Miller, et al. have developed the combination of the PPAM3 plasmid (the packaging-defective helper genome) for expression of retroviral structural proteins together with the LN vector series to make a vector packaging system where the generation of recombinant wild-type retrovirus is reduced to a minimum through the elimination of nearly all sites of recombination between the vector genome and the packaging-defective helper genome (i.e. LN with pPAM3).

In one embodiment, the retroviral vector may be a Moloney Murine Leukemia Virus of the LN series of vectors, such as those hereinabove mentioned, and described further in Bender, et al. (1987) and Miller, et al. (1989). Such vectors have a portion of the packaging signal derived from a mouse sarcoma virus, and a mutated gag initiation codon. The term "mutated" as used herein means that the gag initiation codon has been deleted or altered such that the gag protein or fragments or truncations thereof, are not expressed.

In another embodiment, the retroviral vector may include at least four cloning, or restriction enzyme recognition sites, wherein at least two of the sites have an average frequency of appearance in eukaryotic genes of less than once in 10,000 base pairs; i.e., the restriction product has an average DNA size of at least 10,000 base pairs. Preferred cloning sites are selected from the group consisting of NotI, SnaBI, SalI, and XhoI. In a preferred embodiment, the retroviral vector includes each of these cloning sites. Such vectors are further described in U.S. Patent Application Serial No. 919,062, filed July 23, 1992, and incorporated herein by reference in its entirety.

When a retroviral vector including such cloning sites is employed, there may also be provided a shuttle cloning vector which includes at least two cloning sites which are compatible with at least two cloning sites selected from the group consisting of NotI, SnaBI, SalI, and XhoI located on the retroviral vector. The shuttle cloning vector also includes at least one desired gene which is capable of being transferred from the shuttle cloning vector to the retroviral vector.

The shuttle cloning vector may be constructed from a basic "backbone" vector or fragment to which are ligated one or more linkers which include cloning or restriction enzyme recognition sites. Included in the cloning sites

are the compatible, or complementary cloning sites hereinabove described. Genes and/or promoters having ends corresponding to the restriction sites of the shuttle vector may be ligated into the shuttle vector through techniques known in the art.

The shuttle cloning vector can be employed to amplify DNA sequences in prokaryotic systems. The shuttle cloning vector may be prepared from plasmids generally used in prokaryotic systems and in particular in bacteria. Thus, for example, the shuttle cloning vector may be derived from plasmids such as pBR322; pUC 18; etc.

The vectors include one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and ß-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, TK promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The vectors then are employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, -2, -AM, PA12, T19-14X, VT-19-17-H2, CRE, CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The first and second vectors containing the first and second nucleic acid sequences encoding an agent such that the tumor cells are rendered sensitive to an interaction agent,

and an agent capable of providing for the inhibition, prevention, or destruction of the growth of the tumor cells upon expression of the nucleic acid sequence encoding the agent, wherein such agent is not an agent which renders tumor cells sensitive to an interaction agent, may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation.

The first and second producer cell lines then are administered directly to or adjacent to the tumor in an amount effective to inhibit, prevent, or destroy the growth of the tumor. In general, the first and second producer cell lines are administered in as large a volume as will be tolerated by the host. In general, the first and second producer cell lines are administered in an amount of from about 1×10^6 cells/kg to about 1×10^8 cells/kg of host weight. The exact amount of producer cells to be administered is dependent upon various factors, including but not limited to, the type of the tumor, the location of the tumor, and the size of the tumor. In some cases, repeat administration of the producer cells may be required.

In general, the first and second producer cell lines are administered to the tumor such that the vector particles generated by the first and second producer cell lines are able to transduce the tumor cells. The first and second producer cell lines may be administered directly to or adjacent to the tumor, or to a systemic pathway which enables vector particles generated by the first and second producer cell lines to travel to and transduce tumor cells. For example, the producer cell lines may be injected into the blood stream (i.e., intravenous administration), or into the cerebrospinal fluid in order to treat tumors of the central nervous system. The producer cells also may be

administered intraperitoneally, subcutaneously, or intramuscularly. The exact mode of administration is dependent upon the type of tumor which is treated.

The producer cells may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier such as, for example, a saline solution or a buffer solution or other isomolar aqueous solution.

Upon administration of the first and second producer cell lines to the tumor, the producer cells generate viral vector particles. The viral vector particles then transduce the surrounding tumor cells. Because tumor cells, and in particular cancerous tumor cells, in general are actively replicating cells, the retroviral vector particle would be integrated into and expressed preferentially or exclusively in the tumor cells as opposed to normal cells.

Although the invention has been described with respect to the first nucleic acid sequence being contained in a first expression vector (such as a retroviral vector), and the second nucleic acid sequence being contained in a second expression vector (which also may be a retroviral vector), whereby such vectors are contained in first and second producer cell lines which generate first and second viral vector particles containing the first and second nucleic acid sequences, it also contemplated within the scope of the present invention that the first nucleic acid sequence and the second nucleic acid sequence may be contained in one expression vehicle (such as a retroviral vector). The vector then is transduced into a packaging cell line to form a producer cell line which generates viral vector particles containing the first and second nucleic acid sequences. Such viral vector particles are generated by the producer cell line upon administration of the producer cells to the tumor, whereby such viral vector

particles containing the first and second nucleic acid sequences transduce the tumor cells, and the tumor cells express the proteins encoded by the first and second nucleic acid sequences.

Also, it is contemplated that within the scope of the present invention that the first nucleic acid sequence and the second nucleic acid sequence may be contained in first and second expression vehicles, respectively. The first and second expression vehicles (which may be first and second retroviral vectors, then may be transduced into a single packaging cell line to form a producer cell line which generates first and second viral vector particles containing the first and second nucleic acid sequence, respectively. The first and second viral vector particles, upon administration of the producer cells to the tumor, transduce the tumor cells, and the tumor cells express the proteins encoded by the first and second nucleic acid sequences.

Tumors which may be treated in accordance with the present invention include malignant and non-malignant tumors.

Malignant (including primary and metastatic) tumors which may be treated include, but are not limited to, those occurring in the adrenal glands; bladder; bone; breast; cervix; endocrine glands (including thyroid glands, the pituitary gland, and the pancreas); colon; rectum; heart; hematopoietic tissue; kidney; liver; lung; muscle; nervous system; brain; eye; oral cavity; pharynx; larynx; ovaries; penis; prostate; skin (including melanoma); testicles; thymus; and uterus.

In accordance with the present invention, the agent which renders the tumor cells sensitive to an interaction agent is a negative selective marker; i.e., a material which in combination with a chemotherapeutic or interaction

agent inhibits, prevents, or destroys the growth of the tumor cells.

Thus, upon transduction of the tumor cells with the negative selective marker, an interaction agent is administered to the human host. The interaction agent interacts with the negative selective marker in order to prevent, inhibit, or destroy the growth of the tumor cells.

Negative selective markers which may be employed include, but are not limited to, thymidine kinase, such as Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase; and cytosine deaminase.

In one embodiment, the negative selective marker is a viral thymidine kinase selected from the group consisting of Herpes Simplex Virus thymidine kinase, cytomegalovirus thymidine kinase, and varicella-zoster virus thymidine kinase. When such viral thymidine kinases are employed, the interaction or chemotherapeutic agent preferably is a nucleoside analogue, for example, one selected from the group consisting of ganciclovir and acyclovir. Such interaction agents are utilized efficiently by the viral thymidine kinases as substrates, and such interaction agents thus are incorporated lethally into the DNA of the tumor cells expressing the viral thymidine kinases, thereby resulting in the death of the tumor cells.

In another embodiment, the negative selective marker is cytosine deaminase. When cytosine deaminase is the negative selective marker, a preferred interaction agent is 5-fluorocytosine. Cytosine deaminase converts 5-fluorocytosine to 5-fluorouracil, which is highly cytotoxic. Thus, the tumor cells which express the cytosine deaminase gene convert the 5-fluorocytosine to 5-fluorouracil and are killed. Another interaction agent which may be employed is 1-2-deoxy-2-fluoro-ß-D-arabinofuranosil-5-iodouracil (FIAU).

The interaction agent is administered in an amount effective to inhibit, prevent, or destroy the growth of the transduced tumor cells. For example, the interaction agent may be administered in an amount from 5 mg to 10 mg/kg of host weight per day, depending on overall toxicity to a patient. The interaction agent preferably is administered systemically, such as, for example, by intravenous administration, by parenteral administration, by intraperitoneal administration, or by intramuscular administration.

When producer cells or other expression media including a negative selective marker are administered to a tumor in vivo, a "bystander effect" may result, i.e., tumor cells which were not originally transduced with the nucleic acid sequence encoding the negative selective marker may be killed upon administration of the interaction agent. The "bystander effect" is disclosed in U.S. Patent Application Serial No. 07/877,519, filed May 1, 1992, which is incorporated herein by reference. Although the scope of the present invention is not intended to be limited by any theoretical reasoning, the transformed tumor cells may be producing a diffusible form of the negative selective marker that either acts extracellularly upon the interaction agent, or is taken up by adjacent, non-transformed tumor cells, which then become susceptible to the action of the interaction agent. It also is possible that one or both of the negative selective marker and the interaction agent are communicated between tumor cells.

In one embodiment, the agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells, wherein the agent is not an agent which renders tumor cells sensitive to an interaction agent, and such agent is encoded by the second nucleic acid sequence, is a cytokine. In one embodiment, the cytokine is an interleukin. Other cytokines which may be employed include

interferons and colony-stimulating factors, such as GM-CSF. Interleukins which may be encoded by the second nucleic acid sequence include, but are not limited to, Interleukin-1; Interleukin-1ß; Interleukin-2; Interleukin-3; Interleukin-4; Interleukin-5; Interleukin-6; Interleukin-7; Interleukin-8; Interleukin-9; Interleukin-10; Interleukin-11; and Interleukin-12. In one embodiment, the interleukin is Interleukin-2.

Although the scope of the present invention is not to be limited by any theoretical reasoning, the administration of an expression vehicle (such as a retroviral vector particle), which includes a gene encoding a cytokine, to the tumor cells, enables the expression of the cytokine (such as Interleukin-2, for example) by the tumor cells. The expression of the cytokine may activate the immune system against the tumor and aids in eradicating residual tumor cells not killed by the bystander effect of the negative selective marker.

In addition, Applicants have found unexpectedly that the cytokine (such as Interleukin-2) enhances the bystander effect of the negative selective marker (such as Herpes Simplex thymidine kinase). Thus, there is a synergistic effect provided by the interaction between the cytokine and the negative selective marker.

In a preferred embodiment, a first packaging cell line is transduced with a first retroviral vector, such as those hereinabove described, which includes the Herpes Simplex thymidine kinase gene. A second packaging cell line is transduced with a retroviral vector, such as those hereinabove described, which includes the Interleukin-2 gene. The transduced packaging cell lines (producer cells) are administered in vivo to the tumor in an acceptable pharmaceutical carrier and in an amount effective to inhibit, prevent, or destroy the growth of the tumor. Upon administration of the producer cells to the tumor, the

producer cells generate a first group of viral particles including a gene encoding the negative selective marker, and a second group of viral particles including a gene encoding a cytokine. The two groups of viral particles transduce the tumor cells. The host then is given an agent such as ganciclovir, or 1-2-deoxy-2-fluoro-B-D-arabinofuranosil-5-iodouracil (FIAU), which interacts with the Herpes Simplex Virus thymidine kinase to kill the transduced tumor cells. As hereinabove mentioned, a "bystander effect" also may occur, whereby non-transduced tumor cells also may be killed. In addition, the expression of Interleukin-2 by transduced tumor cells will stimulate an immune response against the tumor and help to kill those tumor cells which were not transduced with the vector particles and not killed as a result of the Herpes Simplex thymidine kinase bystander effect.

The method of the present invention is particularly useful when the targeted tumor is localized in a particular region of the body for extended periods of time, such as, for example, ovarian cancer, which tends to remain localized in the abdomen for extended periods of time; melanoma; renal carcinoma; brain tumors; liver tumors; and head and neck cancer. Because ovarian cancer cells remain localized in the abdomen, the producer cells may be administered intraperitoneally. Such injection of the producer cells also minimizes undesirable propagation of the virus in the body, especially when replication-competent retroviral vectors are used because such vectors are produced continuously.

Because most cells of the body express receptors for amphotropic retroviral vectors, any vector particle which escapes from the local environment of the tumor should immediately bind to another cell. Most cells are not in cycle, however, and therefore will not integrate the genes carried by the vector and will not express any genes which

it contains. Thus, the proportion of potential target cells which are in cycle at the time of exposure will be small, and systemic toxic effects on normal tissues will be minimized.

In accordance with yet another aspect of the present invention, there is provided a method of treating a tumor in a host. The tumor is sensitive to a bystander effect. The method enhances the bystander effect and comprises administering to the tumor a nucleic acid sequence which encodes a first agent which renders the tumor cells sensitive to an interaction agent. A second agent then is administered to the tumor. The second agent provides for the inhibition, prevention, or destruction of the growth of the tumor cells. The second agent is not an agent which renders tumor cells sensitive to an interaction agent. The second agent enhances the therapeutic effect of the interaction agent by an effect independent of the immune system of the host. The tumor then is treated with the interaction agent.

In one embodiment, the nucleic acid sequence encoding the first agent is contained in an expression vehicle, which may be a viral vector such as those hereinabove described. The viral vector is contained in a producer cell line, which is administered to the tumor to produce a virus in an amount effective to transform cells of the tumor. The viral vector may be a retroviral vector as hereinabove described and may be administered in amounts hereinabove described.

In another embodiment, the first agent is a negative selective marker, which may be selected from those hereinabove described, and may be administered in amounts hereinabove described. In yet another embodiment, the second agent is a cytokine, which also may be selected from those hereinabove described.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

Example 1

A. Construction of pG1TkSvNa

The following describes the construction of pG1TkSvNa, a schematic of which is shown in Figure 6. This vector contains the Thymidine Kinase (hTK) gene from herpes simplex virus I regulated by the retroviral promoter and the bacterial gene, neomycin phosphotransferase (Neo^R) driven by an SV40 promoter. The hTK gene confers sensitivity to the DNA analogs acyclovir and ganciclovir, while the Neo^R gene product confer resistance to the neomycin analogue, G418.

To make pGITkSvNa, a three step cloning strategy was used. First, the herpes simplex thymidine kinase gene (Tk) was cloned into the G1 plasmid backbone to produce pG1Tk. Second, the Neo^R gene (Na) was cloned into the plasmid pSvBg to make pSvNa. Finally, SvNa was excised from pSvNa and ligated into pG1Tk to produce pG1TkSvNa.

Plasmid pG1TkSvNa was derived from plasmid PG1 (Figure 3). Plasmid pG1 was constructed from pLNSX (Palmer, et al., Blood, Vol. 73, pgs. 438-445), and incorporated herein by reference. The construction strategy for plasmid pG1 is shown in Figure 1. The 1.6kb EcoRI fragment, containing the 5' Moloney Murine Sarcoma Virus (MoMuSV) LTR, and the 3.0kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used to generate the vector plasmid pG1 (Figure 3) by the insertion of the 1.6kB EcoRI fragment containing the 5' LTR into the unique EcoRI site of pGO. Thus, pG1 (Figure 3) consists of a retroviral

vector backbone composed of a 5' portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender, et al. 1987), a 54 base pair multiple cloning site (MCS) containing, from 5' to 3' the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindII, ApaI, and ClaI and a 3' portion of MoMuLV from base pairs 7764 to 7813 (numbered as described (Van Beveren, et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985), and incorporated herein by reference (Figure 2). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo^r gene, the ß-galactosidase gene, the hygromycin^r gene, and the SV40 promoter.

To construct pBg (Figure 4) the 3.0 kb BamHI/EcoRI lacZ fragment that encodes β-galactosidase was isolated from pMC1871 (Pharmacia). This fragment lacks the extreme 5' and 3' ends of the β-galactosidase open reading frame. Linkers that would restore the complete lacZ open reading frame and add restriction sites to each end of the lacZ gene were synthesized and ligated to the BamHI/EcoRI lacZ fragment. The structure of the 5' linker was as follows: 5' - 1/2 NdeI - SphI - NotI - SnaBI - SalI - SacII - AccI - NruI - BgIII - III 27 bp ribosomal binding signal - Kozak consensus sequence/NcoI - first 21 bp of the lacZ open reading frame - 1/2 BamHI - 3'. The structure of the 3' linker was as follows: 5' - 1/2 mutated EcoRI - last 55 bp of the lacZ open reading frame - XhoI - HindIII - SmaI - 1/2 EcoRI - 3'. The restriction sites in the linkers were chosen because they are not present in the neomycin resistance gene, the β-galactosidase gene, the hygromycin resistance gene, or the SV40 promoter. The 27 bp ribosomal binding signal was included in the 5' linker because it is believed to enhance mRNA stability (Hagenbuchle, et al., Cell 13:551-563, 1978 and Lawrence and Jackson, J. Mol. Biol. 162:317-334, 1982), both of

which are incorporated herein by reference. The Kozak consensus sequence (5'-GCCGCCACCATGG-3') has been shown to signal initiation of mRNA translation (Kozak, Nucl.Acids Res. 12:857-872, 1984), incorporated herein by reference. The Kozak consensus sequence includes the NcoI site that marks the ATG translation initiation codon.

pBR322 (Bolivar et al., Gene, 2:95, 1977), incorporated herein by reference was digested with NdeI and EcoRI and the 2.1 kb fragment that contains the ampicillin resistance gene and the bacterial origin of replication was isolated. The ligated 5' linker - lacZ - 3' linker DNA described above was ligated to the pBR322 NdeI/EcoRI vector to generate pBg. pBg has utility as a shuttle plasmid because the lacZ gene can be excised and another gene inserted into any of the restriction sites that are present at the 5' and 3' ends of the lacZ gene. Because these restriction sites are reiterated in the pG1 plasmid, the lacZ gene or genes that replace it in the shuttle plasmid construct can easily be moved into pG1.

A 1.74 kB BglII/PvuII fragment containing the Herpes Simplex Virus Type I thymidine kinase gene (GenBank accession no. V00467, incorporated herein by reference) was excised from the pX1 plasmid (Huberman, et al., Exptl. Cell Res. Vol. 153, pgs 347-362 (1984) incorporated herein by reference), blunted with the large (Klenow) fragment of DNA polymerase I, and inserted into the unique SnaBI site in the pG1 multiple cloning site, to form plasmid pG1TK. (Figure 5).

A 339 bp PvuII/HindIII SV40 early promoter fragment obtained from the plasmid pSV2Neo (Southern et al, Journal of Molecular and Applied Genetics 1:327-341(1982)), incorporated herein by reference, was then inserted into pBg in the unique NruI site to generate the plasmid pSvBg (Figure 5). The pSvBg plasmid was digested with BglII/XhoI to remove the lacZ gene, and the ends were made blunt using

the Klenow fragment. An 852 bp EcoRI/AsuII fragment containing the coding sequence of the neomycin resistance gene was removed from pN2 (Armentano, et al., J. Virol., Vol. 61, pgs. 1647-1650 (1987) and incorporated herein by reference), blunted with Klenow fragment and ligated into the 2.5 kb blunted BglII/XhoI fragment generated hereinabove, resulting in pSvNa. The SV40 promoter/neomycin resistance gene cassette was then removed from pSvNa as a 1191bp SalI/HindIII fragment. The pG1Tk plasmid was then digested with SalI/HindIII and ligated with the SV40/neo^r fragment to generate pG1TkSvNa. (Figure 6).

B. Generation of Producer Cell Line

A producer cell line was made from vector plasmid and packaging cells. The PA317/G1TkSvNa producer cell was made by the same general techniques used to make previous clinically relevant retroviral vector producer cell lines. The vector plasmid pG1TkSvNa DNA was transfected into a ecotropic packaging cell line, PE501. Supernatant from the PE501 transfected cells was then used to transinfect the amphotropic packaging cell line (PA317). Clones of transinfected producer cells were then grown in G418 containing medium to select clones that contain the Neo^R gene. The clones were then titered for retroviral vector production. Several clones were then selected for further testing and finally a clone was selected for clinical use.

5 x 10^5 PE501 cells (Miller, et al., Biotechniques, Vol. 7, pgs. 980-990 (1989), incorporated herein by reference) were plated in 100 mm dishes with 10 ml high glucose Dulbecco's Modified Essential Medium (DMEM) growth medium supplemented with 10% fetal bovine serum (HGD10) per dish. The cells were incubated at 37°C, in 5% CO_2/air overnight.

The plasmid pG1TKSvNa then was transfected into PE501 cells by $CaPO_4$ precipitation using 50 μg of DNA by the following procedure.

50 μg of DNA, 50 μl 10 x $CaCl_2$, and 450μl of sterile H_2O was mixed in a 15 ml polypropylene tube to yield a 0.25M Ca Cl_2 solution containing 50 μg DNA, 0.5 ml 2x BBS (containing 50 mM N-N-bis- (2-hydroxyethyl)- 2-aminoethane-sulfonic acid, 280 mM Na Cl, 1.5 mM Na_2 HPO_4, and 50 mM Hepes, pH6.95). The DNA solution then was left at room temperature for about 20 minutes to 1 hour. The dishes then were incubated at 35°C in a 3% CO_2 atmosphere overnight.

A culture dish(es) with optimum precipitate following the overnight incubation then was (were) selected. The dish(es) then was (were) washed again with PBS to remove the salt and the salt solution. 10 ml of HGD10 medium then was added to the dish(es), and the dish(es) incubated at 37°C in a 5% CO_2 atmosphere for about 48 hrs.

After 48 hours, supernatant was collected from the transfected cells. The dish(es) then was (were) rinsed with 5 ml PBS. The PBS then was removed, and cells were removed with trypsin-EDTA. Serial dilutions of the cells were then inoculated into six 100 mm dishes in medium containing HGD10 and 0.8 mg/ml G418.

The six plates of cells were examined daily. The medium was changed as needed to remove dead cells. Live cells or colonies were allowed to grow to a size such that the colonies are large enough to clone (i.e., the colonies are visible to the naked eye). PE501 ecotropic containing supernatants from such colonies of PE501 cells were collected in volumes of from about 5 to 10 ml, placed in cryotubes, and frozen in liquid nitrogen at -70°C.

PA317 cells (Miller et al. Mol. Cell. Biol. 6:2895-2902 (1986)) then were plated at a density of 5 x 10^4 cells per 100 mm plate on Dulbecco's Modified Essential Medium

(DMEM) including 4.5 g/l glucose, glutamine supplement, and 10% fetal bovine serum (FBS).

The PE501 supernatant then was thawed, and 8 μg/ml of polybrene was added to the supernatant. The medium was aspirated from the plates of PA317 cells, and 7 to 8 ml of viral supernatant was added and incubated overnight.

The PE501 supernatant then was removed and the cells refed approximately 18-20 hours with fresh 10% FBS. One day later, the medium was changed to 10% FBS and G418 (800 μg/ml). The plate then was monitored, and the medium was changed to fresh 10% FBS and G418 to eliminate dying or dead cells as necessary. The plate was monitored for at least 10 to 14 days for the appearance of G418 resistant colonies.

Cloning rings were placed around all selected colonies. The cells were tyrpsinized and incubated into wells in a six well dish in 5 ml of HGD10 plus 1x hypoxanthine aminopterin thymidine (HAT).

If the clones grew to confluency, they were trypsinized and incubated in a 100 ml dish. As a clone in the 100 ml dish approached confluency, its amphotropic vector-containing supernatant was removed and centrifuged at 1,200 to 1,500 rpm for 5 minutes to pellet out cells.

Supernatants were aliquoted into six cryovials (1 ml/vial) and stored in liquid nitrogen. 5 ml of PBS were added to the dish, and the cells were rinsed, and refed with HGD-10 and frozen in 1 ml aliquots with 10% DMSO in liquid nitrogen. The different clones were monitored to determine the one with the highest titer of retroviral vector.

The clone with the highest titer, designated as producer cell line PA317/G1TkSvNa.53, was used to produce a master cell bank.

C. Preparation of pG1TK1SvNa

The plasmid pG1TK1SvNa (Figure 8) was prepared according to the schematic representation shown in Figure 7. It was prepared to remove the partial open reading frame from pG1TKSvNa (Figure 6).

Generation of pSPTK5':

DNA from the plasmid pG1NaSvTk was digested with restriction enzymes BgIII and SmaI and the 1163 base pair (bp) Herpes thymidine kinase (TK) fragment was fractionated by agarose gel electrophoresis and isolated. This fragment contains 56 bp of the TK 5'-untranslated region and 1107 bp of the TK translation open reading frame. The 1163 bp TK fragment was ligated to the plasmid vector pSP73 (Promega Corporation, Madison, WI) that had been digested with restriction enzymes BgIII and SmaI. The resulting ligated plasmid construct was named pSPTK5' because it contains the 5' portion of the TK open reading frame but lacks the last 21 bp of the open reading frame and the translation termination codon.

PCR of the TK open reading frame:

pG1NaSvTK plasmid DNA was linearized by digesting it with BgIII. The linearized pG1NaSvTK was used as a template for polymerase chain reaction (PCR) using a forward primer that contains the first 17 bases of the TK open reading frame (5'-GCACCATGGCTTCGTACCCCTGC-3') and a reverse primer that contains complementary sequence for an XhoI site, the TK translation termination codon, and the last 19 bp of the TK open reading frame (5'-CCTGCATCGATTCTCGAGTCAGTTAGCCTCCCCCATCTCC-3'). 30 cycles of PCR were performed as follows: 1 minute at 94°C and 2 minutes at 60°C with a final 7 minute extension cycle at 72°C. PCR products were fractionated on an agarose gel and the expected 1215 bp fragment that includes the full-length TK open reading frame was isolated. The isolated fragment was digested with restriction enzymes PstI and XhoI, digestion products were fractionated on an agarose gel, and

the 420 bp fragment was isolated. This fragment extends from the PstI site at the nucleotides encoding amino acids 249-250 of the TK open reading frame through the XhoI site immediately downstream of the TGA translation termination codon.

Generation of pSPTK1:

pSPTK5' was digested with PstI and the 3993 bp fragment that contains the pSP73 vector and the 5' portion of the TK open reading frame was isolated following agarose gel electophoresis. This 3993 bp fragment was ligated to the PCR-generated 420 bp PstI/XhoI fragment that contains the 3' end of the TK open reading frame (above). Ligated plasmid DNA was transformed into E. coli DK5α competent cells (Gibco/BRL, Gaithersburg, MD) and DNA from ampicillin-resistant colonies was screened by restriction enzyme digestion. Plasmids that appeared to contain the full-length TK open reading frame were termed pSPTK1. The DNA from several pSPTK1 clones was dideoxy sequenced in the region from the PstI site through the XhoI site (the region that was generated by PCR). pSPTK1 clone #4 was found to match the expected TK sequence in this region and was used for construction of pG1TK1SvNa.

Generation of pG1TK1SvNa:

pSPTK1 DNA was digested with BgIII and the 5' overhanging ends were repaired by incubation of the digested DNA with deoxy nucleotides and Klenow fragment of E. coli DNA polymerase I. The DNA was then digested with XhoI to generate a 1225 bp fragment that contains 56 bp of TK 5'-untranslated region and the full-length TK open reading frame. This blunt/XhoI fragment was ligated to pG1XSvNa DNA that had been digested with SnaBI and SalI.

To construct pG1XSvNa, the 1.2 kb SvNa fragment was excised from pSvNa (Part A above) with SalI and HindIII. This fragment was ligated to pG1 that had been digested

with SalI and HindIII. The ligated plasmid was termed pG1XSvNa where the "X" denotes a multiple cloning region.

The product DNA from the pG1XSvNa and TK ligation was transformed in DH5α and DNA from ampicillin-resistant colonies was screened as previously described. Plasmids that appeared to contain the TK fragment by diagnostic restriction enzyme digestion were termed pG1TK1SvNa. (Figure 8.) Clone #2 was dideoxy sequenced from the beginning of the 5'-LTR through the end of the 3'-LTR and was found to contain the intact TK open reading frame.

pG1TK1SvNa was used to produce a producer cell by combination with PA317 by the hereinabove described method (Part B above). Such producer cell line was designated as producer cell line PA317/G1SvNa.7.

D. Construction of pG1I2SvNa.

pG1 was cut with HindIII and SalI. pSvNa (Figure 9), which contains the SV40 promoter from pLNSX and the neo^r gene from pN2, was also cut with HindIII and SalI, and a HindIII-SalI fragment containing an SV40 promoter and a β-galactosidase gene was ligated into HindIII/SalI digested pG1 to form pG1XSvNa (Figure 10).

pG1XSvNa was cut at the SnaBI site and a BglII-ClaI restriction fragment containing the Interleukin-2 leader sequence, and Kozak region, Interleukin-2 secretion signal added by oligomers; and the mature Interleukin-2 coding sequence from ATCC with the 3' untranslated region removed, was ligated into the cut pG1XSvNa to form pG1I2SvNa. (Figure 11).

pG1I2SvNa was used to produce a producer cell line by combination with PA317 by the method described in Part B hereinabove. The producer cell line is sometimes hereinafter referred to as PA317/G1I2SvNa.5.

E. Construction of pG1I2GSvNa

pG1XSvNa was cut at the SnaBI site, and a BglII-HindIII restriction fragment containing the Interleukin-2

leader sequence, Kozak region, and a PCR-generated full coding sequence using the Roche Interleukin-2 gene as a template was ligated into the cut pG1XSvNa to form pG1I2GSvNa.11. (Figure 12.)

pG1I2GSvNa was used to produce a producer cell line by combination with PA317 by the method described in Part B hereinabove. The producer cell line is sometimes hereinafter referred to as PA317/G1I2GSvNa.

F. Administration of Herpes Simplex Thymidine kinase producer cells and/or Interleukin-2 producer cells to Athymic Nude Mice

68 athymic nude female mice were injected with 15-30 x 10^6 OVCAR-3 ovarian cancer cells in 1 or 1.5 ml of Hanks BSS solution into the peritoneal cavity. The mice then were divided into four groups. Group A included 19 mice and each mouse received 10 x 10^6 PA317/G1TKSvNa.53 producer cells intraperitoneally 7 days after the injection of the OVCAR-3 cells. Group B included 15 mice and each mouse received 10 x 10^6 PA317/G1I2SvNa.5 producer cells intraperitoneally 7 days after injection of the OVCAR-3 cells. Group C included 18 mice and each mouse received intraperitoneal injections of 10 x 10^6 PA317/G1TKSvNa.53 producer cells and 10 x 10^6 PA317/G1I2SvNa.5 producer cells 7 days after the injection of the OVCAR-3 cells. Group D included 16 mice and each mouse received intraperitoneal injections of 2 x 10^6 PA317/G1TKSvNa.53 producer cells and 2 x 10^6 PA317/G1I2SvNa producer cells 7 days after the injection of the OVCAR-3 cells. Seven days after the injection of the producer cells, 9 mice in Group A, 6 mice in Group B, 13 mice in Group C, and 11 mice in Group D received ganciclovir (GCV) in an amount of 5 mg/kg intraperitoneally and twice daily for 14 days. The other mice received no treatment. All the mice then were evaluated for tumor growth. The number of tumor-free mice in each group is given in Table I below.

TABLE I

Group	Mice free of tumors	
	No GCV Treatment	GCV Treatment
A	0/10	5/9
B	1/9	0/6
C	0/5	12/13
D	0/5	9/11

As shown in Table I, 9 of the 11 mice which were given 2 x 10^6 Herpes Simplex thymidine kinase producer cells and 2 x 10^6 Interleukin-2 producer cells and treated with ganciclovir were found to be free of tumors. On the other hand, only 5 out of 9 mice which were given 10 x 10^6 Herpes Simplex thymidine kinase producer cells and treated with ganciclovir were found to be free of tumors. Such results indicate that there is a synergistic tumoricidal effect between Herpes Simplex thymidine kinase and Interluekin-2. Such synergy also was unexpected in that the nude mice employed in this experiment are immune deficient. Thus, Interleukin-2 unexpectedly increases the bystander effect of the Herpes Simplex thymidine kinase, and such increase in the bystander effect does not require an activity of the immune system.

Example 2

Thirty C57BL black 6-8 week female mice each received a subcutaneous injection of 2 x 10^5 transduced MCA205 fibrosarcoma cells. The mice were divided into 3 groups with 10 mice in each group. Group I received MCA205 cells transduced with vector particles generated from pG1TKSvNa. Group II received MCA205 cells transduced with vector particles generated from pG1I2SvNa. Group III received 1 x 10^5 MCA205 cells transduced with vector particles generated from pG1TKSvNa and 1 x 10^5 MCA205 cells transduced with pG1I2SvNa. Five days after the mice were injected with the MCA205 cells, 5 mice in each group were treated daily with

5 mg/kg of ganciclovir (GCV) intraperitoneally. The median time for tumor regression was recorded. The results are given in Table II below.

Table II

Group to Regression for treated	Tumor Regression (No. of animals) GCV Treatment	No GCV Treatment	Mean Time GCV- Animals
I days	5/5	0/5	25
II days	5/5	0/5	25
III days	5/5	0/5	10

In the animals which did not receive ganciclovir therapy, the average size of the tumors transduced with the Interleukin-2 gene were 32% smaller than the tumors transduced with the Herpes Simplex thymidine kinase gene. All animals that received ganciclovir therapy had complete resolution of their tumors. The animals that received both Herpes Simplex thymidine kinase and Interleukin-2 transduced tumors had significantly decreased times to complete tumor resolution. Thus, the two genes in combination induced more rapid tumor destruction with ganciclovir therapy. In addition, one possible explanation of the fact that complete tumor regression of the tumors transduced with Interleukin-2 alone upon administration of ganciclovir may be that cytokines such as Interleukin-2 may activate expression of endogenous thymidine kinase genes in the tumor cells. The induction would have to be

surprisingly large because the endogenous enzyme is unable to use ganciclovir as a substrate significantly, if at all.

Example 3

Human OVCAR-3 cells were transduced with vector particles generated from either pG1TKSvNa or pG1I2SvNa, and selected in 1.0 mg/ml G418 for 7-14 days.. Cells surviving the G418 selection were used as the transduced tumor cells in this example.

At Day 1, OVCAR-3 cells were added to the wells of a microtiter plate at 10,000 cells/well in a total volume of 100 μl of RPMI1640 with 10% fetal calf serum. Each well had a different proportion (in percent) of wild type OVCAR-3 cells; OVCAR-3 cells transduced with vector particles generated from pG1TKSvNa; and OVCAR-3 cells transduced with vector particles generated from pG1I2SvNa. At Day 2, 100 μl of ganciclovir was added to each well at a concentration of 2.5 μg/ml, 5.1 μg/ml or 12.5 μg/ml. The cells were cultured for 44 to 48 hours in an incubator at 37°C with 5% CO_2. 0.5 μCi of ^{3}H-thymidine then was added to each well in a volume of 20 μl into each well of the plate. Four to eight hours later, the cells were harvested and radioactivity was measured as counts per minute (cpm). The cpm is directly proportional to the proliferation rate in this assay. The percent decrease in proliferation was measured for each well. The results are given in Table III below.

Table III

% Wild-Type Decrease in OVCAR-3 Proliferation	% Hstk- transduced OVCAR-3	% IL-2 transduced OVCAR-3	%
0	100	0	96
50	50	0	91
75	25	0	10
90	10	0	0
100	0	0	0
0	0	100	98
50	0	50	92
75	0	25	50
90	0	10	0
100	0	0	0
50	25	25	97
95	2.5	2.5	89
50	10	40	96
95	1	4	85

As shown in the above table, Interleukin-2 and ganciclovir induce a bystander effect in this assay that is nearly comparable to the Herpes Simplex thymidine kinase effect alone. When the Herpes Simplex thymidine kinase and Interleukin-2 transduced cells are cultured together, the bystander effect is potentiated. This suggests that the in vivo synergy between the Herpes Simplex thymidine kinase and Interleukin-2 may not be simply an enhancement of the immune response, but may include an unexplained mechanism whereby Interleukin-2 directly or indirectly affects the anti-tumor response to ganciclovir. Such mechanism does not include an activity of the immune system.

Example 4

The procedure of Example 3 was repeated with respect to A375 melanoma cells. The percent decrease in proliferation of cells in each well containing various proportions of wild-type and transduced cells is given in Table IV below.

TABLE IV

% Wild-Type Decrease in A375 Proliferation	% Hstk- transduced A375	% IL-2 transduced A375	%
0	100	0	99
50	50	0	96
75	25	0	88
90	10	0	62
95	5	0	47
98	2	0	23
99	1	0	26
100	0	0	0
0	0	100	0
50	0	50	36
75	0	25	31
90	0	10	33
95	0	5	34
98	0	2	0
99	0	1	0
100	0	0	0
50	25	25	98
75	12.5	12.5	93
95	2.5	2.5	48
97.5	1.25	1.25	36
50	10	40	96

75	5	20	83
87.5	2.5	10	56
95	1	4	27

Example 5

The procedure of Example 3 was repeated with respect to 786-0 renal carcinoma cells. The percent decrease in proliferation of cells in each well containing various proportions of wild-type and transduced cells is given in Table V below.

TABLE V

% Wild-Type Decrease in 786-0 Proliferation	% Hstk- transduced 786-0	% IL-2 transduced 786-0	%
0	100	0	96
50	50	0	91
75	25	0	81
90	10	0	42
95	5	0	28
98	2	0	22
99	1	0	0
100	0	0	0
0	0	100	100
50	0	50	100
75	0	25	100
90	0	10	100
95	0	5	100
98	0	2	100
99	0	1	100
100	0	0	100
50	25	25	93
75	12.5	12.5	81
87.5	6.25	6.25	70

95	2.5	2.5	54
97.5	1.25	1.25	15
50	10	40	87
75	5	20	79
87.5	2.5	10	33
95	1	4	48
97.5	0.5	2	7

Example 6

Fifteen women, all 18 years of age and older, and suffering from recurrent or progressive epithelial ovarian cancer localized in the abdominal cavity, and who failed standard therapy for the disease, each are given a Tenckhoff catheter intraperitoneally. At least two days after the placement of the catheter, each patient begins the treatment cycle. The cycle is begun by administering to each patient PA317/G1TK1SvNa.7 producer cells and PA317/G1I2GSvNa.11 producer cells through the Tenckhoff catheter over a period of 4 hours. The total volume of fluid administered is between 1 and 4 liters and the producer cells are administered as a 50:50 mixture of cells at a concentration of from about $2x10^6$ cells/ml to about $10x10^6$ cells/ml. The total number of producer cells administered per treatment cycle is about $1x10^{10}$ cells.

Fourteen days after the patients receive the producer cells, each patient receives an intravenous dose of 5 mg/kg of ganciclovir daily for 14 days. After the 14-day period of ganciclovir treatment, the patients receive no treatment for 7 days to end the treatment cycle. At the end of the 7-day period without ganciclovir treatment, the treatment cycle is repeated.

The disclosure of all patents, publications (including published patent applications), and database entries referenced in this specification are specifically

incorporated by reference in their entirety to the same extent as if each such individual patent, publication, database entry were specifically and individually indicated to be incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. A method of treating a tumor in a host, wherein said tumor is sensitive to a bystander effect, by enhancing said bystander effect comprising:

(a) administering to said tumor a first nucleic acid sequence, said first nucleic acid sequence encoding an agent which renders the tumor cells sensitive to an interaction agent;

(b) administering to said tumor a second nucleic acid sequence which encodes an agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells, wherein said agent is not an agent which renders tumor cells sensitive to an interaction agent, wherein said agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells, and wherein said agent is not an agent which renders tumor cells sensitive to an interaction agent, enhances the therapeutic effect of said interaction agent by an effect independent of the immune system of said host; and

(c) treating said tumor with said interaction agent.

2. The method of Claim 1 wherein said first nucleic acid sequence and said second nucleic acid sequence are contained in at least one expression vehicle.

3. The method of Claim 2 wherein said at least one expression vehicle is a first expression vehicle including said first nucleic acid sequence and a second expression vehicle including said second nucleic acid sequence.

4. The method of Claim 3 wherein each of said first expression vehicle and said second expression vehicle is a viral vector, and said first viral vector is contained in a first producer cell line and said second viral vector is contained in a second producer cell line, wherein said first producer cell line and said second producer cell line

are administered to the tumor, and wherein said first producer cell line produces a first virus and said second producer line produces a second virus, wherein said first virus and said second virus are produced in amounts effective to transform cells of said tumor.

5. The method of Claim 1 wherein said first nucleic acid sequence encodes a negative selective marker.

6. The method of Claim 5 wherein said negative selective marker is selected from the group consisting of Herpes Simplex Virus thymidine kinase; cytomegalovirus thymidine kinase; and varicella-zoster virus thymidine kinase.

7. The method of Claim 6 wherein said interaction agent is selected from the group consisting of ganciclovir and acyclovir.

8. The method of Claim 7 wherein said interaction agent is ganciclovir.

9. The method of Claim 1 wherein said second nucleic acid sequence encodes a cytokine.

10. The method of Claim 9 wherein said cytokine is an interleukin.

11. The method of Claim 10 wherein said interleukin is selected from the class consisting of Interleukin-1; Interleukin-1ß; Interleukin-2; Interleukin-3; Interleukin-4; Interleukin-5; Interleukin-6; Interleukin-7; Interleukin-8; Interleukin-9; Interleukin-10; Interleukin-11; and Interleukin-12.

12. The method of Claim 11 wherein said interleukin is Interleukin-2.

13. The method of Claim 4 wherein each of said first viral vector and said second viral vector is a retroviral vector.

14. The method of Claim 4 wherein said first producer cell line is administered in an amount of from about 1 x 10^6 cells/kg to about 1 x 10^8 cells/kg of host weight.

15. The method of Claim 4 wherein said second producer cell line is administered in an amount of from about 1 x 10^6 cells/kg to about 1 x 10^8 cells/kg of host weight.

16. The method of Claim 7 wherein said interaction agent is administered in an amount of from about 5 mg/kg to about 10 mg/kg of host weight per day.

17. The method of Claim 1 wherein said tumor is ovarian cancer.

18. A composition comprising:

a first producer cell including a first viral vector, said first viral vector including a first nucleic acid sequence encoding an agent which renders tumor cells sensitive to an interaction agent; and

a second producer cell including a second viral vector, said second viral vector including a second nucleic acid sequence which encodes an agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells, wherein said agent is not an agent which renders tumor cells sensitive to an interaction agent, wherein said agent which provides for the inhibition, prevention, or destruction of the growth of tumor cells, and wherein said agent is not an agent which renders tumor cells sensitive to an interaction agent, enhances the therapeutic effect of said interaction agent by an effect independent of the immune system.

19. The composition of Claim 18 wherein said first nucleic acid sequence encodes a negative selective marker.

20. The composition of Claim 19 wherein said negative selective marker is selected from the group consisting of Herpes Simplex Virus thymidine kinase; cytomegalovirus thymidine kinase; and varicella-zoster virus thymidine kinase.

21. The composition of Claim 18 wherein said second nucleic acid sequence encodes a cytokine.

22. The composition of Claim 21 wherein said cytokine is an interleukin.

23. The composition of Claim 22 wherein said interleukin is selected from the class consisting of Interleukin-1; Interleukin-1ß; Interleukin-2; Interleukin-3; Interleukin-4; Interleukin-5; Interleukin-6; Interleukin-7; Interleukin-8; Interleukin-9; Interleukin-10; Interleukin-11; and Interleukin-12.

24. The composition of Claim 23 wherein said interleukin is Interleukin-2.

25. The composition of Claim 18 wherein each of said first viral vector and said second viral vector is a retroviral vector.

26. A method of treating a tumor in a host, wherein said tumor is sensitive to a bystander effect, by enhancing said bystander effect, comprising:

(a) administering to said tumor a nucleic acid sequence, said nucleic acid sequence encoding a first agent which renders the tumor cells sensitive to an interaction agent;

(b) administering to said tumor a second agent which provides for the inhibition, prevention, or destruction of the growth of the tumor cells, wherein said second agent is not an agent which renders tumor cells sensitive to an interaction agent, wherein said second agent enhances the therapeutic effect of said interaction agent by an effect independent of the immune system of said host; and

(c) treating said tumor with said interaction agent.

27. The method of Claim 26 wherein said nucleic acid sequence encoding said first agent is contained in an expression vehicle.

28. The method of Claim 27 wherein said expression vehicle is a viral vector, and said viral vector is contained in a producer cell line, wherein said producer cell line is administered to the tumor, wherein said

producer cell line produces a virus, wherein said virus is produced in an amount effective to transform cells of said tumor.

29. The method of Claim 26 wherein said first agent is a negative selective marker.

30. The method of Claim 29 wherein said negative selective marker is selected from the group consisting of Herpes Simplex Virus thymidine kinase; cytomegalovirus thymidine kinase; and varicella-zoster virus thymidine kinase.

31. The method of Claim 30 wherein said interaction agent is selected from the group consisting of ganciclovir and acyclovir.

32. The method of Claim 31 wherein said interaction agent is ganciclovir.

33. The method of Claim 26 wherein said second agent is a cytokine.

34. The method of Claim 33 wherein said cytokine is an interleukin.

35. The method of Claim 34 wherein said interleukin is selected from the class consisting of Interleukin-1; Interleukin-1ß; Interleukin-2; Interleukin-3; Interleukin-4; Interleukin-5; Interleukin-6; Interleukin-7; Interleukin-8; Interleukin-9; Interleukin-10; Interleukin-11; and Interleukin-12.

36. The method of Claim 35 wherein said interleukin is Interleukin-2.

37. The method of Claim 28 wherein said viral vector is a retroviral vector.

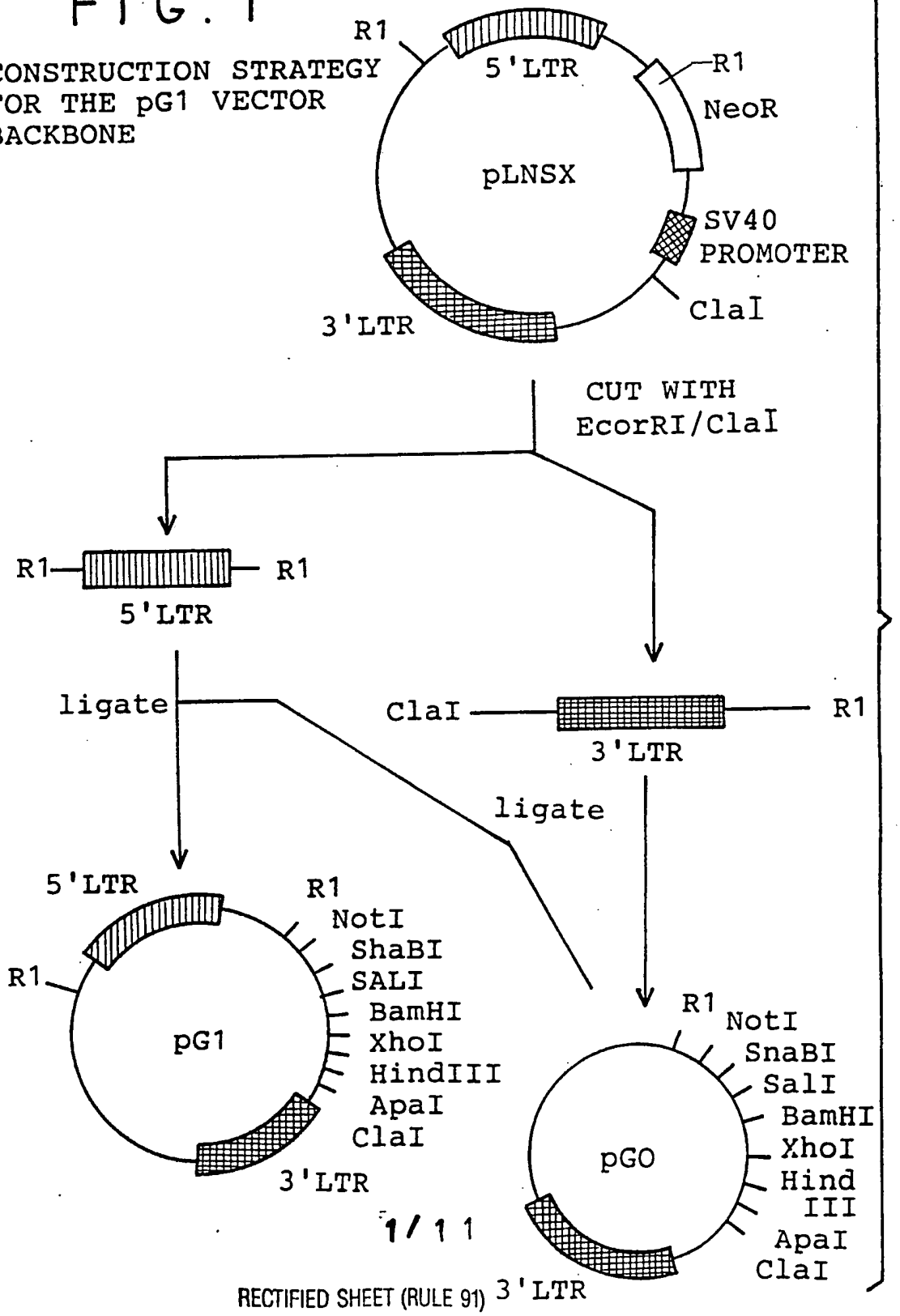
38. The method of Claim 28 wherein said producer cell line is administered in an amount of from about 1×10^6 cells/kg to about 1×10^8 cells/kg of host weight.

39. The method of Claim 31 wherein said interaction agent is administered in an amount of from about 5 mg/kg to about 10 mg/kg of host weight per day.

40. The method of Claim 26 wherein said tumor is ovarian cancer.

FIG. 1

CONSTRUCTION STRATEGY FOR THE pG1 VECTOR BACKBONE



SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID

1/2 EcoRI	NotI	SnaBI	SalI	BamHI	XhoI	HindIII	ApaI
AATTC	GCGGCCGC	TACGTA	GTCGTA	GGATCC	CTCGAG	AAGCTT	GGGCCC
G	CGCCGGCG	ATGCAT	ATGCAT	CCTAGG	GAGCTC	TTCGAA	CCCGGG

1/2ClaI

AT

TAGC

FIG. 2

FIG. 3

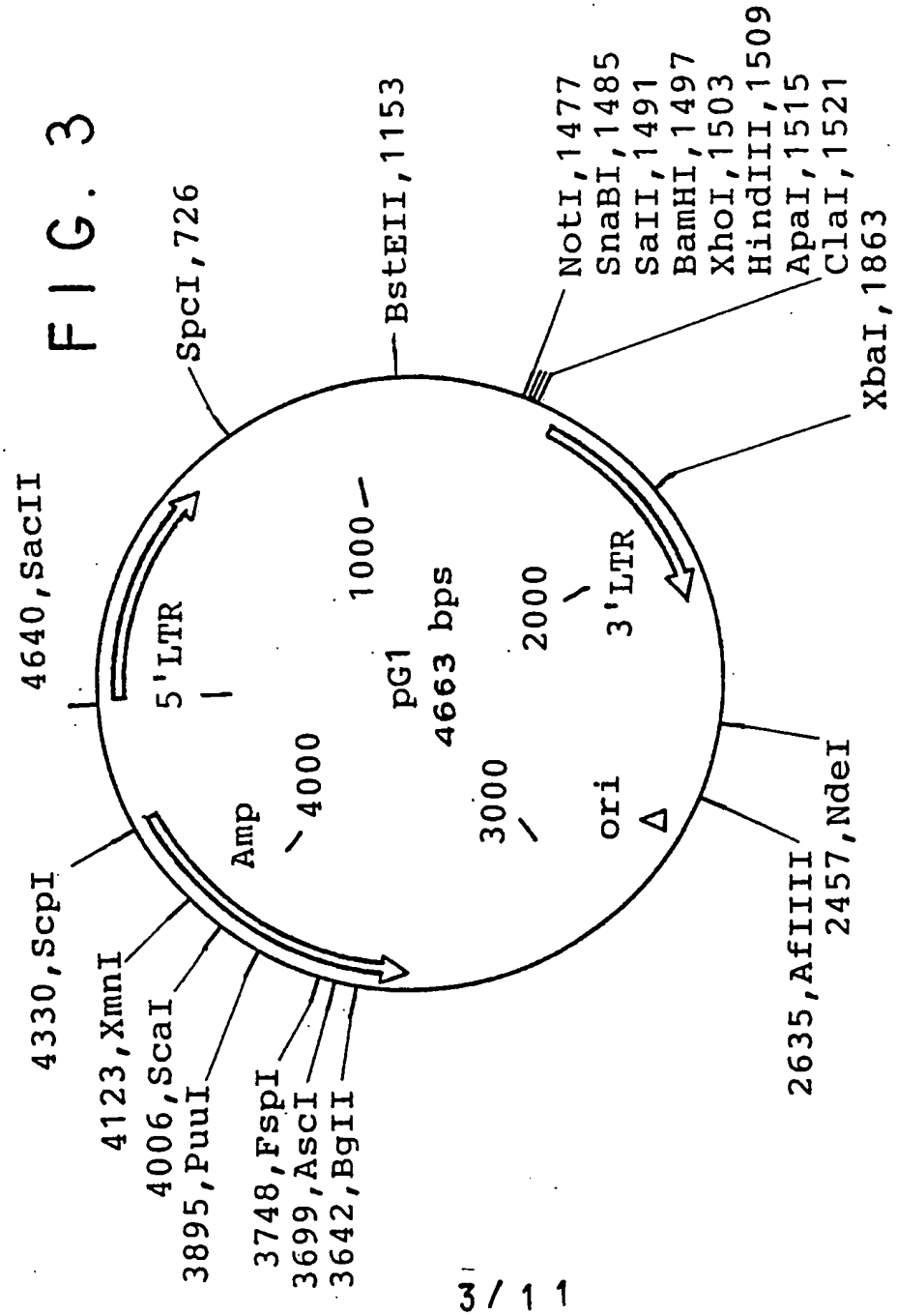
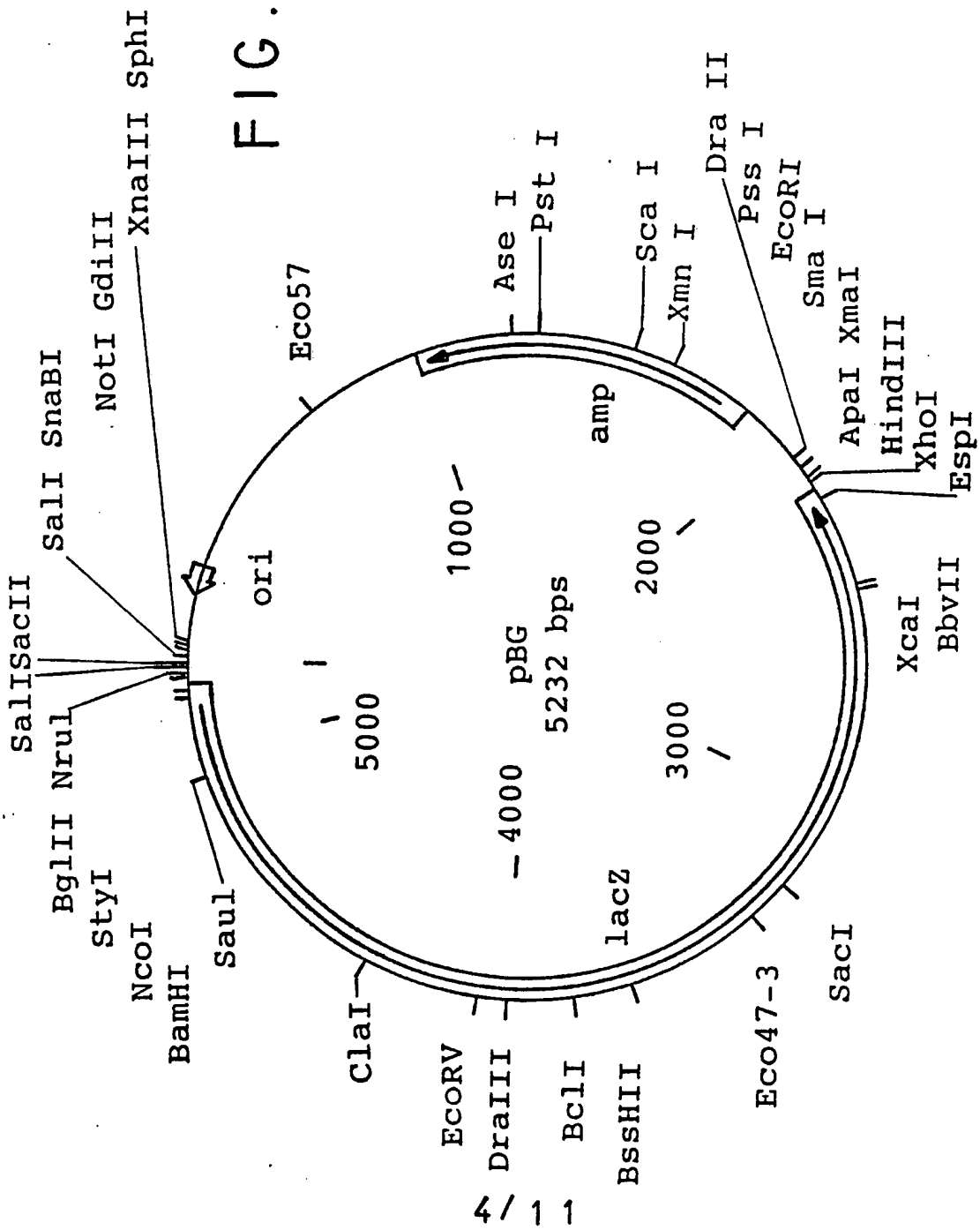


FIG. 4



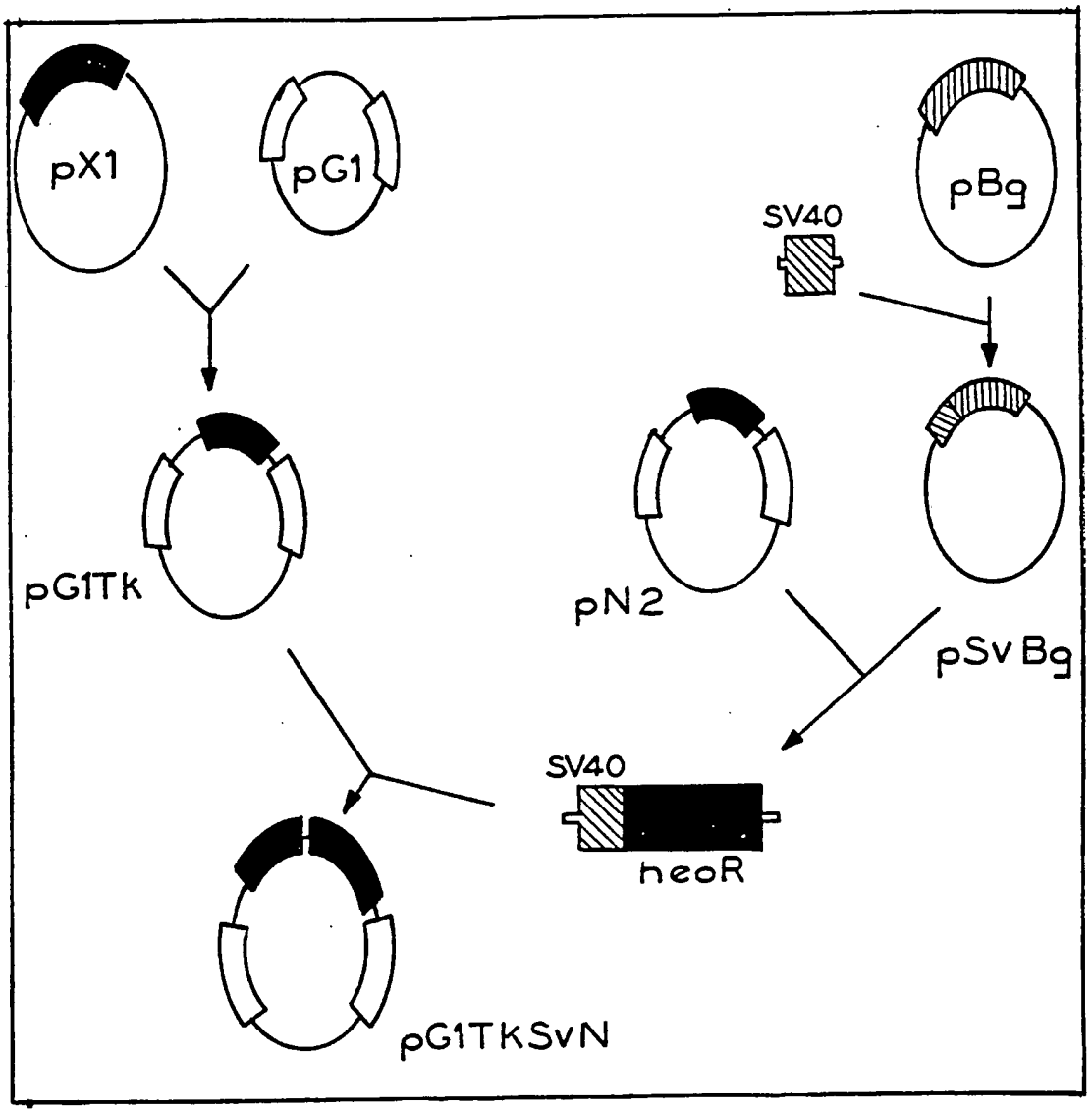
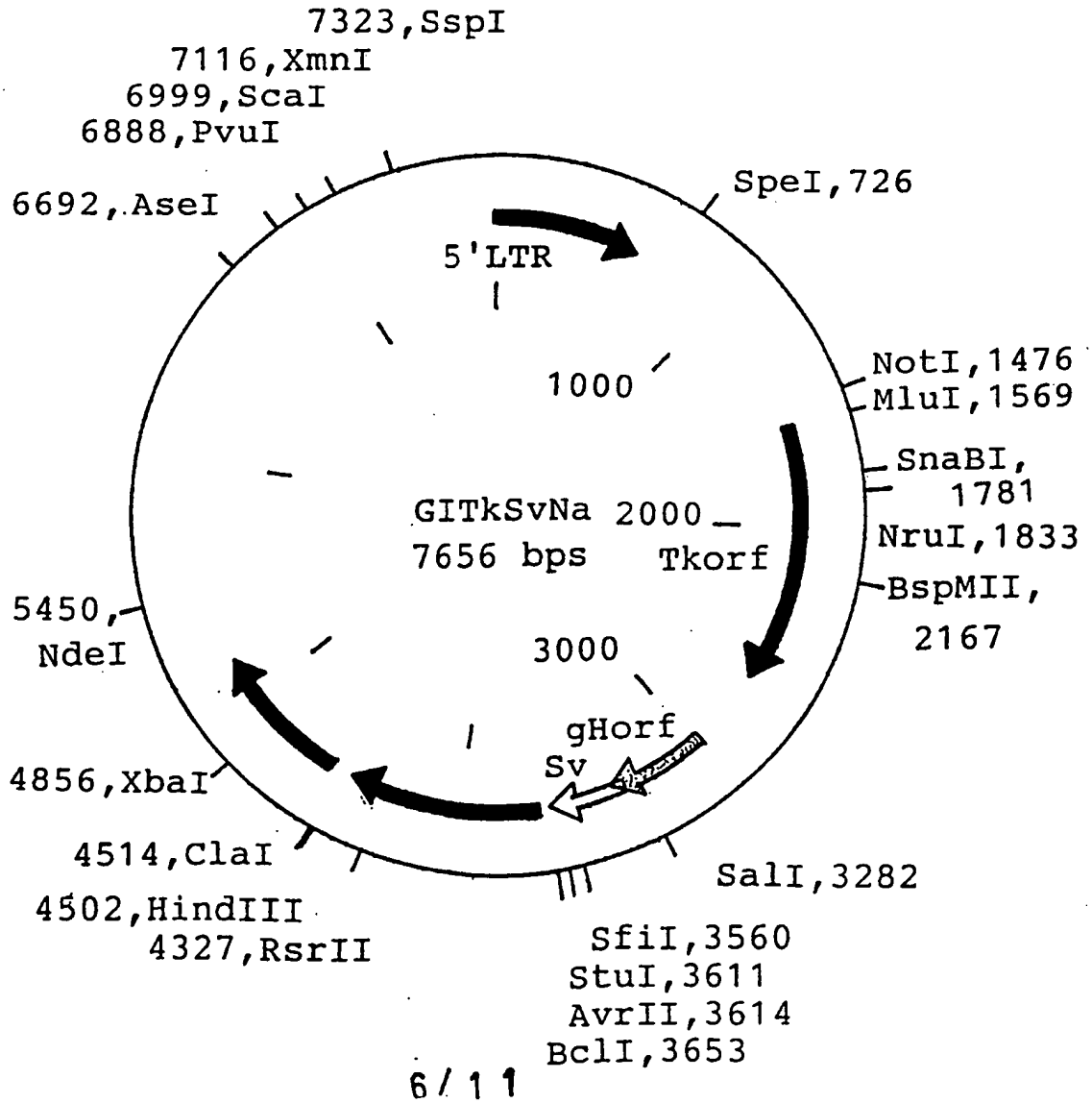
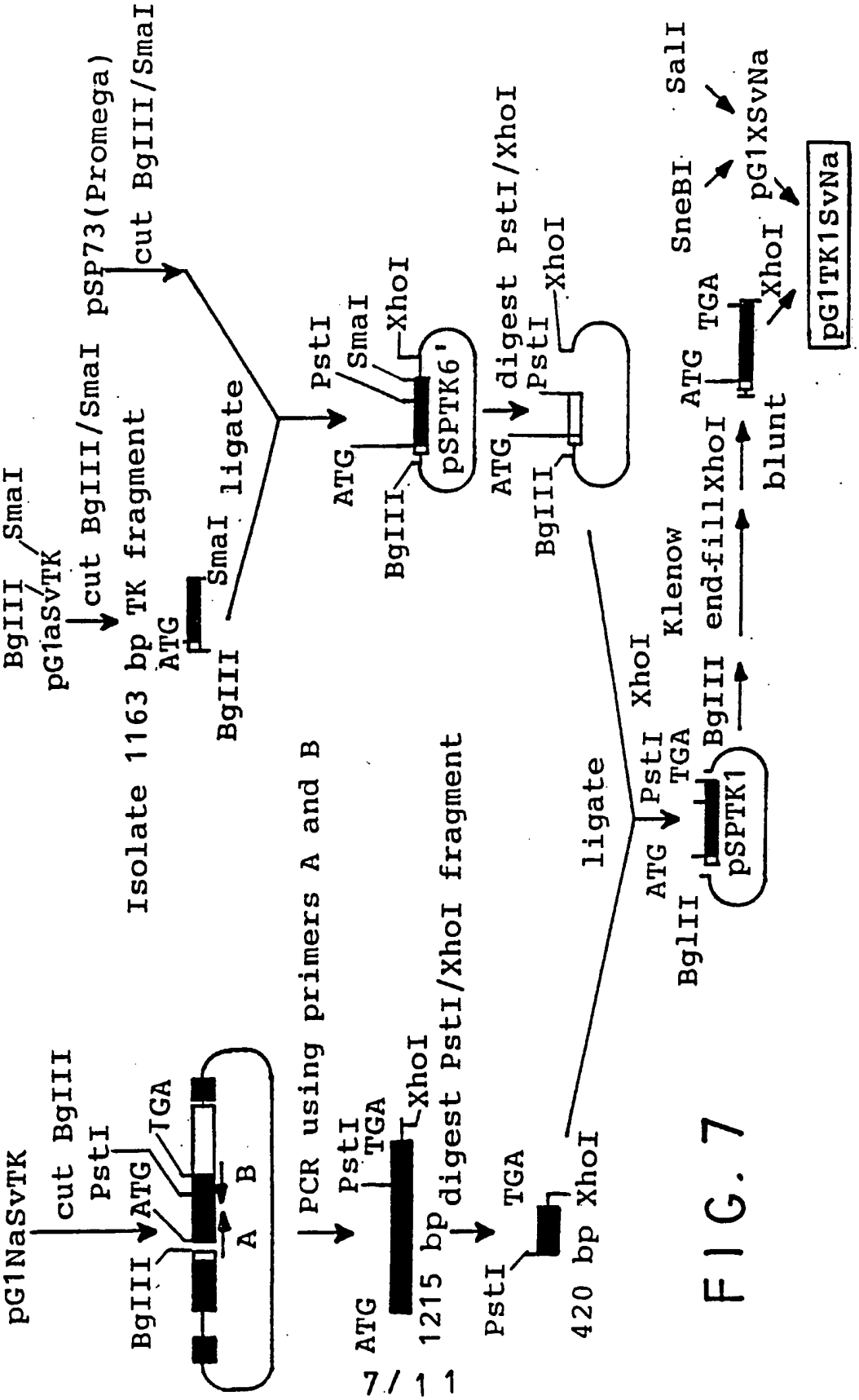


FIG. 5

RECTIFIED SHEET (RULE 91)

FIG. 6




Generation of TK1 Vectors
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cut BglII
PstI
ATG
TGA
BglII
A
B
PCR using primers A and B
PstI
TGA
XhoI
ATG
1215 bp
digest PstI/XhoI fragment
PstI
TGA
420 bp XhoI
ligate
PstI XhoI
TGA
ATG
BglII
pSPTK1
Klenow
BglII end-fill XhoI
ATG
TGA
XhoI
blunt
SneBI
SalI
pG1XSvNa
pG1TK1SvNa
BglII
SmaI
pG1aSvTK
cut BglII/SmaI
Isolate 1163 bp TK fragment
ATG
BglII
SmaI ligate
pSP73(Promega)
cut BglII/SmaI
PstI
SmaI
XhoI
ATG
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PstI
XhoI
BglII

FIG. 7

7/11

FIG. 8

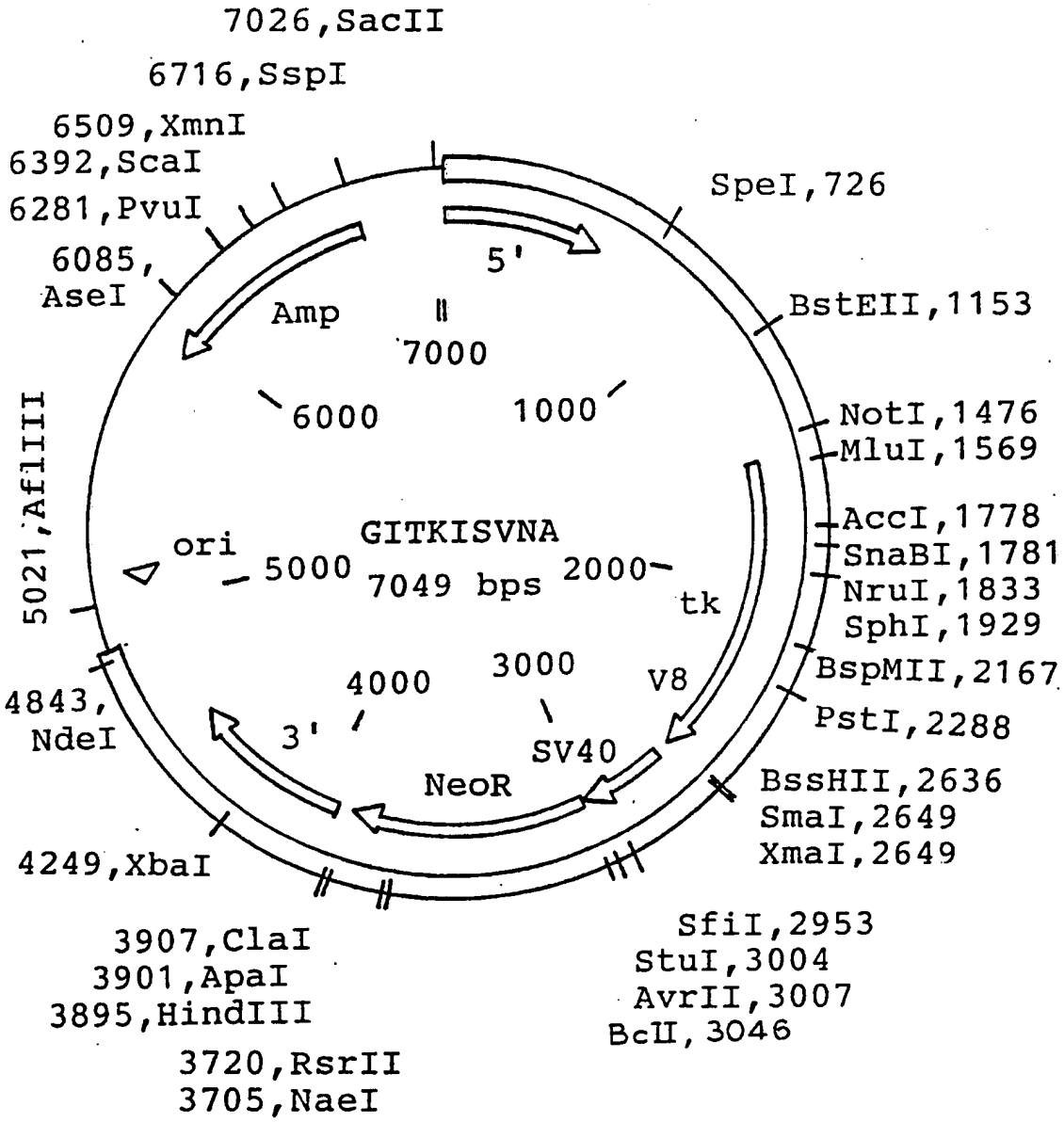


FIG. 9

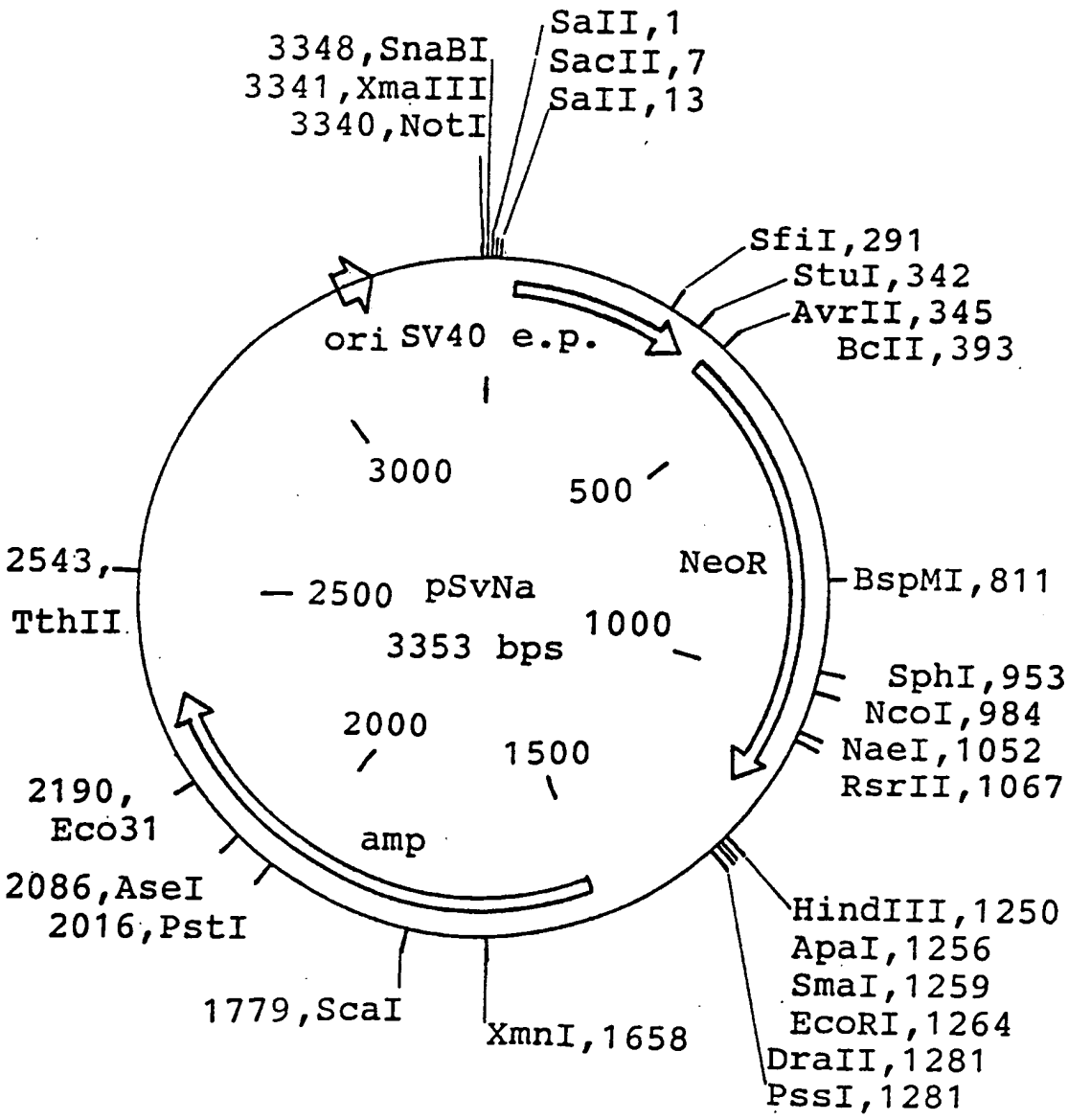


FIG. 10

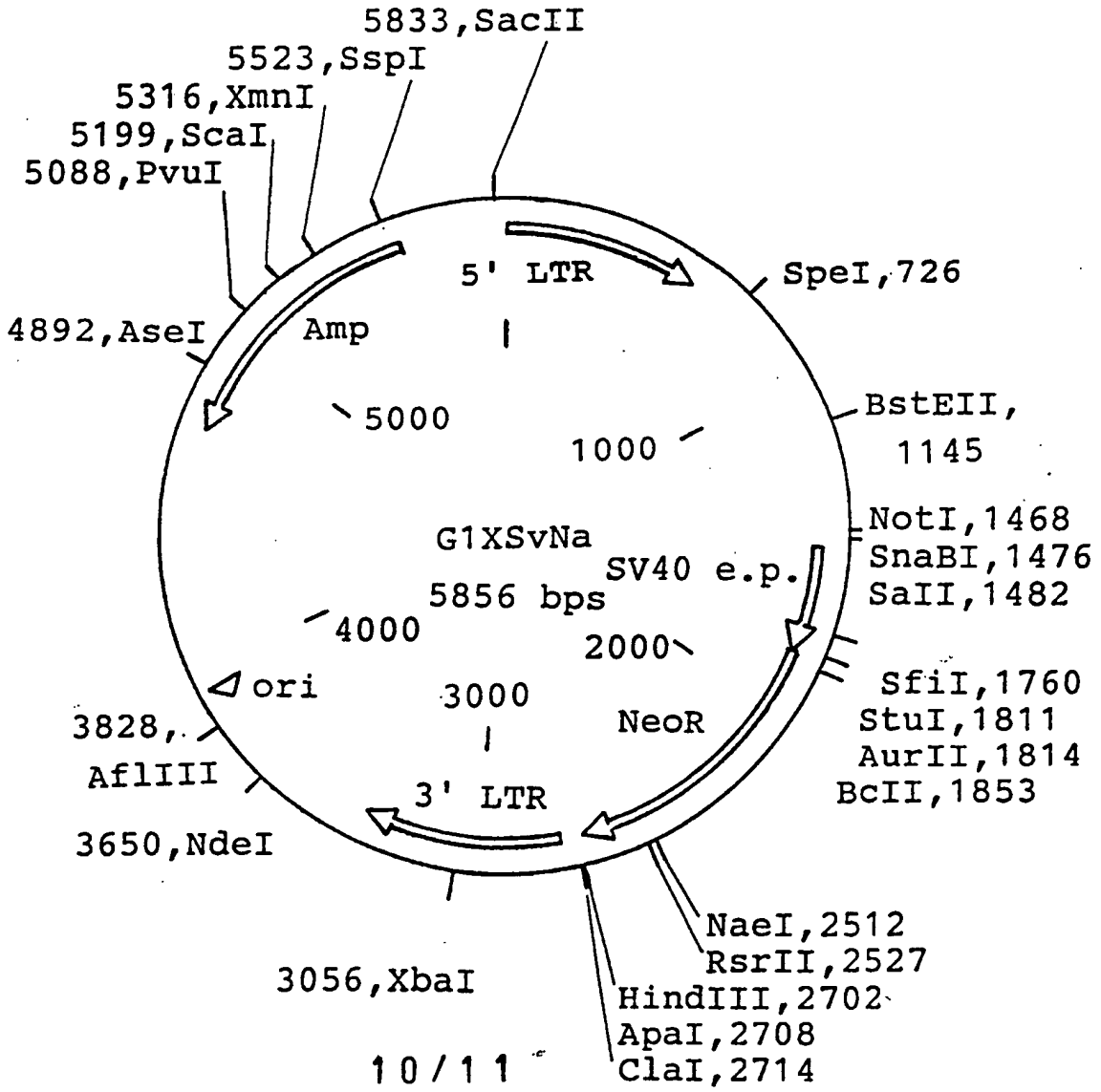
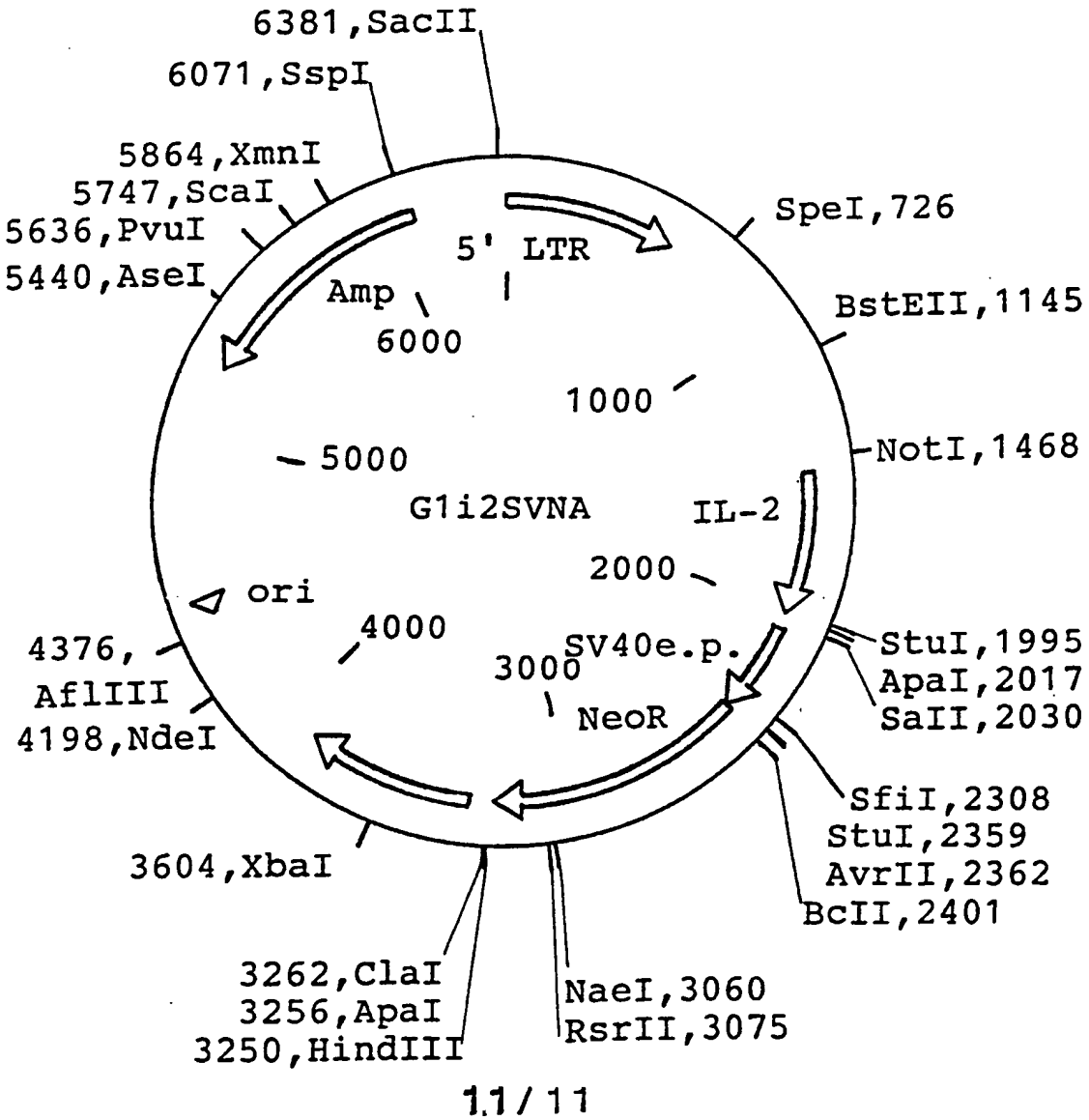


FIG. 11



INTERNATIONAL SEARCH REPORT

Int...ational application No.
PCT/US94/11251

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :514/44; 424/93.21; 435/172.3, 240.2, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/93.21; 435/172.3, 240.2, 320.1; 935/62

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, APS, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 53, issued 01 January 1993, Ram et al, "In Situ Retroviral-Mediated Gene Transfer for the Treatment of Brain Tumors in Rats", pages 83-88, see entire document.	1-40
Y	EP, A, 0,476,953 (MARTUZA ET AL.) 25 March 1992, see entire document.	1-40
Y	Science, Volume 256, issued 12 June 1992, Culver et al, "In Vivo Gene Transfer with Retroviral Vector-Producer Cells for Treatment of Experimental Brain Tumors", pages 1550-1552, see entire document.	1-40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
28 DECEMBER 1994	JAN 19 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JACQUELINE STONE Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11251

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Exp. Med., Volume 172, issued October 1990, Gansbacher et al, "Interleukin 2 Gene Transfer into Tumor Cells Abrogates Tumorigenicity and Induces Protective Immunity", pages 1217-1224, see entire document.	1-40
Y	J. Exp. Med., Volume 177, issued May 1993, Minasi et al, "The Selective Ablation of Interleukin 2-producing Cells Isolated from Transgenic Mice", pages 1451-1459, see entire document.	1-40
Y	Proc. Natl. Acad. Sci. USA, Volume 85, issued October 1988, Borrelli et al, "Targeting of an Inducible Toxic Phenotype in Animal Cells", pages 7572-7576, see entire document.	1-40
Y	Science, Volume 254, issued 01 November 1991, Golumbek et al, "Treatment of Established Renal Cancer by Tumor Cells Engineered to Secrete Interleukin-4", pages 713-716, see entire document.	1-40
Y	WO, A, 93/04167 (BARBA ET AL) 04 March 1993, see entire document.	1-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11251

A. CLASSIFICATION OF SUBJECT MATTER:
IPC:(6)

IPC(6): A61K 48/00; C12N 15/86, 5/22, 5/16, 15/26, 15/25, 15/24, 15/52, 15/38